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*Iowa State University*

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ADHERENCE-ASSOCIATED PROPERTIES OF MORAXELLA BOVIS

*Iowa State University*

Ph.D. 1984

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Adherence-associated properties of Moraxella bovis

by

Susan Herkender Jackman

A Dissertation Submitted to the  
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For the Graduate College

Iowa State University

Ames, Iowa

1984



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#### EXPLANATION OF THESIS FORMAT

This thesis consists of a general introduction including objectives, literature review, three separate manuscripts, a general conclusion, references and acknowledgements. The doctoral candidate, Susan Herkender Jackman, is the senior author and principal investigator for each of the manuscripts.

## GENERAL INTRODUCTION

Infectious bovine keratoconjunctivitis (IBK) is a contagious ocular disease of cattle that is characterized by lacrimation, inflammation, and corneal ulceration or opacity (1,105). Most evidence indicates that Moraxella bovis is the primary etiologic agent of IBK (1,69).

Little is known concerning the pathogenesis of IBK. Reports of the histological changes which occur have been provided from the study of lesions in naturally occurring episodes of IBK (10,105) and in calves experimentally infected with M. bovis (9,10). Certain virulence factors such as pili (8,66) and hemolysin (68) have been associated with pathogenicity. It has also been suggested that certain factors or environmental conditions predispose the eye to infection with M. bovis (1,20,22, 45,46,85).

On ocular epithelial surfaces, as on other tissues in which there is mechanical clearance by cell sloughing or fluid movement, persistence is a central concept to bacterial pathogenesis. In many cases, persistence of an organism may involve adhesion.

Over the past several years, the adherence of bacteria to mammalian tissue surfaces has attracted a great deal of interest and has gained increasing importance as an initial event in the pathogenesis of microbial infections. There are numerous reports in the literature describing the adherence of bacteria to specific tissue surfaces but the exact mechanistic basis of bacterial adherence in the colonization and the development of disease has been more difficult to elucidate. This is mainly due to the

complexity of the interaction between bacteria and host cell surfaces and the difficulty in assessing information from in vivo adherence models or in designing truly representative in vitro models.

In general, the approach for studying bacterial adherence to host cells has utilized in vitro systems. Mammalian cells (suspensions of cells, monolayers of tissue culture cells, explanted tissue) are incubated with bacteria. After a given time period, unattached bacteria are removed (filtration, washing, differential centrifugation) and the adhered bacteria are quantitated (visualization, radioactivity determinations). These in vitro systems have both advantages and disadvantages. The main advantage being the ability to control the experimental situation and to evaluate the information obtained, while the disadvantage lies in the interpretation of in vitro data with respect to its in vivo significance. Recently, Freter and Jones (19) have discussed the role of adherence in the pathogenesis of bacterial infections and have again suggested that in vitro models alone cannot establish any particular bacterial adhesin as the necessary entity for colonization or virulence. They emphasize the point that truly to understand adherence and colonization, in vivo animal models are required. Regardless of its limitation, in vitro tests have contributed to the identification of bacterial adhesins, host cell receptors, and adherence mechanisms of bacteria.

The objective of this research was to study certain aspects in the pathogenesis of IBK that involved the adherence of M. bovis to bovine ocular epithelium. The investigation included a description of pili by electron microscopy and hemagglutination development of in vitro assays

to measure adherence of M. bovis to intact corneal and conjunctival epithelium and suspensions of epithelial cells, measurement of surface hydrophobicity by hydrophobic interaction chromatography, and assessment of the effect of Mycoplasma bovoculi preinfection on the adherence of M. bovis to bovine ocular epithelium.

## LITERATURE REVIEW

In the literature, there has been some attempt to define and standardize the terminology used in discussing bacterial adherence (2,42). The term adhesin is generally used to denote the structure on a bacterial cell that mediates the attachment of certain bacteria to a surface. The term receptor describes the structures that bind adhesins. Adherence and adhesion are used to define the process of attachment of bacteria to surfaces. Finally, the terms fimbriae or pili denote specific types of adhesins and usually refer to morphology. Some (2,42) have suggested that fimbriae and pili be further differentiated, such that only fimbriae is used to describe bacterial attachment and pili is reserved for the description of transfer of genetic information. However, due to the popularity of both words (fimbriae and pili) investigators will probably continue to use these terms interchangeably.

### Physiochemical Factors in Bacterial Attachment

Most descriptions of the physiochemical factors of bacterial adherence have been based on theories of long and short range attractive forces between two objects. Long and some short range forces in bacterial adherence are explained by nonspecific physiochemical interactions. Other short range forces are based on the assumption that a specific receptor molecule on a host cell is recognized by a specific molecule on the bacterium.

### Long range forces

Long range attractive forces between bacteria and eukaryotic cells have been suggested to function according to the DLVO<sup>1</sup> theory. Basically, the theory states that the energy of interaction between two charged bodies of like sign is the sum of the energy of repulsion and the energy of attraction between the two bodies. At long distances of separation, the energy of attraction is greater than the energy of repulsion. The attraction is considered weak and reversible. When the distance of separation becomes shorter, repulsive forces are dominant but if these repulsive forces are overcome and the separation distance becomes even shorter, then the forces of attraction are again greater and the binding of the two bodies can be maximal and irreversible. With respect to the very close range attractive forces, these are more adequately described by specific adhesin-receptor interactions and will be discussed later.

Discussions of the DLVO theory with respect to Neisseria (101) and to bacteria in general (42) have been provided in other reviews. Two observations concerning bacterial adherence to mammalian cells need to be stated because they emphasize the applicability of the DLVO theory in describing interactions of a long-range nature. First, both the

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<sup>1</sup>The theory's name is the initials of the authors who studied the interaction between negatively charged particles (Derjaguin and Landau; Verwey and Overbeck).



bacterial and eukaryotic cell surface are negatively charged; this fulfills a basic principle of the theory. Secondly, both attractive and repulsive forces decrease as the radius of curvature of the bodies decrease, but the repulsive forces do so at a faster rate. Therefore, it is considered advantageous for bacteria to approach a eukaryotic cell with adhesins of small radii, such as fimbriae or pili.

#### Short range nonspecific forces

Short range nonspecific attractive forces are more aptly described by mechanisms other than those of the DLVO theory. In the past few years, hydrophobic properties of bacterial surfaces have been suggested to contribute to short range attraction between bacteria and host surfaces. They have been investigated using a variety of species of microorganisms. Hydrophobicity can be measured by a number of methods which include: partition between aqueous polymer two-phase systems (94), hydrophobic interaction chromatography (92), adherence to hydrocarbons (82), adherence to polystyrene (81), precipitation of cells by a salting out procedure (51), and the partition of a radiolabeled fatty acid between a cell surface and a buffer solution (44,56).

Interest was drawn to the study of surface hydrophobicity when it was observed that the degree of hydrophobicity correlated with other properties of bacteria. With Escherichia coli, reports have shown an association between hemagglutinating activity and hydrophobic characteristics. Some demonstrated that mannose-resistant hemagglutination

correlated with hydrophobicity (51,92,100) while others indicated that bacteria with mannose-sensitive hemagglutinating ability were more hydrophobic (38,63). Association between mannose-resistant hemagglutinating characteristics and surface hydrophobicity also has been shown for Yersinia enterocolitica (18).

Studies with Salmonella typhimurium 395MS (a smooth strain) and lipopolysaccharide defective mutants (rough strains) derived from the parent strain showed that the reduction of the O-side chain and core oligosaccharides of the rough mutants correlated with increased cell surface hydrophobicity (32,52,55,56). A similar study with Proteus mirabilis also demonstrated that rough mutants have a higher surface hydrophobicity than smooth strains (84).

The effect of growth at different pH values and relative surface hydrophobicity was studied with Neisseria gonorrhoeae (53). Growth at pH 6.0 appeared to decrease hydrophobicity when compared to growth at pH 7.2. Interpretation of results was complicated however by the observation that piliated organisms tended to reduce hydrophobic interactions at pH 7.2, whereas the reverse was found with growth at pH 6.0. Further work with Neisseria has demonstrated that the level of iron in the growth medium can also have a complex effect on hydrophobicity in association with fimbriation and possession of colony opacity-associated (COA) proteins (54). When bacteria were grown in limiting iron concentrations, piliation had little effect on hydrophobicity while variants lacking COA proteins were distinctly more hydrophobic than variants possessing these proteins. In iron excess, piliated variants became less

hydrophobic while nonpiliated organisms and those having COA proteins were not affected.

Malmqvist (56) reported changes in hydrophobicity during bacterial growth of Staphylococcus aureus strain V8. The hydrophobicity increased during the exponential growth-phase of the culture while in the stationary growth-phase there was a loss of hydrophobicity. Similarly Olsson et al. (64) observed lower hydrophobicity of a Streptococcus mutans strain in late stationary phase growth under conditions where the pH of the medium was continuously adjusted to 6.8. Westergren and Olsson (104) noted a decreased hydrophobicity in S. mutans and in one strain of Streptococcus sanguis after repeated subculture on blood agar.

The main purpose in studying surface hydrophobic characteristics of bacteria has been to attempt to relate this property with adherence of microorganisms to surfaces. For many bacterial species, possession of a particular adhesin has been correlated with increased hydrophobicity when compared with organisms lacking the adhesin.

Investigations comparing M protein-positive variants of two strains of Streptococcus pyogenes suggested that both M protein and lipoteichoic acid (LTA) contribute to hydrophobic properties (98). Johnson et al. (39), using a M protein-deficient group A streptococcus, extracted T protein free of LTA and demonstrated that it also may contribute to weak hydrophobic bonds and may take part in adherence of group A streptococci to host cells. A mutant of S. sanguis FC-1, was recently isolated that was deficient in its ability to adsorb to hexadecane (24). When examined by electron microscopy, the mutant appeared also to be defective in the

synthesis of fimbriae when compared with S. sanguis FC-1 which possesses fimbriae.

Recent studies with thirteen strains of Y. enterocolitica have also shown good correlation between the presence of fimbriae and high surface hydrophobicity (18).

Extensive work with E. coli has demonstrated the association of adhesive factors with hydrophobic characteristics. Fimbriated E. coli from porcine (51,92,100), bovine (30,51,100) and human (51,100) isolates were more hydrophobic when compared to organisms lacking the specific adhesive antigen. There also have been reports of correlation between the presence of type 1 pili of E. coli and hydrophobicity (51,63).

In contrast, with N. gonorrhoeae (97) and Neisseria meningitidis (96) piliation of the organisms did not seem to contribute to hydrophobicity and with Serratia marcescens (32) nonfimbriated cells were more hydrophobic than fimbriated counterparts.

In many reports, hydrophobicity was associated with adherence to a eukaryotic cell type (38,52,67,83,100) or in the case of oral bacteria, adherence to saliva-coated hydroxyapatite (23,58,104).

#### Short range specific forces

Although there is a general correlation between hydrophobicity of bacterial strains and their adherence to eukaryotic cells or experimentally prepared salivary pellicles, it seems unlikely that hydrophobic interactions, or any nonspecific attractive force, alone can account for the highly specific way in which bacteria attach to mammalian tissues.

There is much evidence to suggest that bacteria have on their surfaces adhesins capable of binding in a specific manner to complementary receptors on host tissue cell surfaces (40). To demonstrate the specificity of the interaction, several approaches have been employed:

- (1) the bacteria or host cell can be treated with enzymes or chemicals to remove, alter, or block the structures participating in adherence,
- (2) the adhesin or receptor can be blocked by specific antibody produced against these structures, or (3) the isolated adhesin or receptor can be used to inhibit competitively the interaction.

The first approach was used most often in the initial attempts to identify bacterial adhesins and mammalian receptors and to define their specificity. The use of carbohydrates to inhibit bacterial adherence to mammalian cells has been widespread and the subject has been discussed in several reviews (40,42,61,65,90). In summary, it is thought that the bacteria attach to the sugar residues which compose at least part of the host cell surface receptor. Mannose and its derivatives have been shown to inhibit attachment of some E. coli strains to epithelial cells (62) but has most often been used to describe receptors for attachment on erythrocytes (17,27) and to classify adhesins, especially pili, according to hemagglutination patterns (17). L-fucose, as well as D-mannose have been suggested as host cell receptor moieties for erythrocytes and intestinal epithelial cells for Vibrio cholerae (41). It has been reported that D-galactosides are recognized by Pseudomonas aeruginosa on human erythrocytes (26) and by N. gonorrhoeae on buccal epithelial cells (65). Certain Mycoplasma sp. (14,78,79), P. aeruginosa

(77), and E. coli possessing fimbriae (50) have been suggested to utilize sialic acid receptors on epithelial cells. Carbohydrates, both in glycoprotein as well as in glycolipid forms, also have been suggested to be present in the epithelial cell and erythrocyte receptor for E. coli and the evidence has been reviewed by Gaastra and de Graaf (21). Evidence has been presented that the eukaryotic surface receptor for Pasteurella multocida (29) on rabbit pharyngeal cells and HeLa cells and for Shigella flexneri (37) on guinea pig colonic cells may have a carbohydrate component. A recent report has demonstrated that the receptor on buccal cells for N. meningitidis is probably not a simple carbohydrate but a complex type of oligosaccharide (96). Recent studies have suggested that fibronectin, a glycoprotein found on the surfaces of epithelial cells, may act as a receptor for the binding of S. pyogenes (4).

Using similar approaches, bacterial adhesins have been identified with respect to their chemical nature. Fimbrial adhesins in general (42) as well as adhesins on Mycoplasma sp. (14,78,79) have been found to be proteinaceous materials. The binding sites of S. mutans are considered to consist of both protein and carbohydrate molecules (25,31) while those of S. pyogenes are identified as lipoteichoic acid (3) with possible involvement of M protein and T protein (39). Evidence also has been presented to suggest that lipoteichoic acid mediates adherence of S. aureus to buccal cells (7).

Currently, the most elegant approach with respect to the specificity of adhesins has been to isolate the particular moiety in purified form. The material is then used to inhibit competitively the binding of the

bacteria to a host cell or to produce antibody against the adhesin. The use of specific antibody has identified fimbrial adhesins for adherence to mammalian cells for E. coli (15,36,48,57,87), S. typhimurium (48), P. aeruginosa (106), and N. gonorrhoeae (6,99). Antibody to a surface protein, PII, of a nonpiliated strain of N. gonorrhoeae also suggested that PII mediates adherence to HeLa cells (95). Similarly, antibody produced against M-like protein, a cell wall component analogous to the M protein of group A streptococci, inhibited adherence of Streptococcus equi to epithelial cells (93) and may be considered to be part of the adhesin for this organism.

Purified adhesin in competitive inhibition studies has confirmed the evidence that pili are the adherence factors for E. coli (36), P. aeruginosa (106), and Pseudomonas fluorescens (80) and has suggested that M-like protein contributes to the adherence of S. equi (93).

Information on the specificity of receptors on mammalian cells by the methods of isolation and purification is lacking in the literature. One report (16) has outlined the isolation and identification of a pilus-specific receptor for E. coli 987. A receptor-containing fraction was released from stored intestinal brush borders of rabbits and exhibited the same specificity in adherence assays as that of intact epithelial cells. Sensitivity of the receptor material to periodate oxidation and pronase digestion was consistent with earlier evidence that certain E. coli receptors are glycoproteins.

A new method for demonstrating the specificity of a particular moiety for mediating adherence of bacteria to host cells has recently been described. It has involved the transfer of genetic material

encoding for pili into a nonadherent organism and correlating the transfer of adherence with the expression of pili. Hull et al. (35) have reported the preparation and transfer of plasmids carrying pili genes from a urinary tract infection E. coli isolate to a nonpiliated E. coli K-12 organism. The newly formed derivatives were found to express functional pili and to possess the adherence properties of the original isolate.

A similar study by Cheney et al. (13) described the conjugal transfer of adherence ability from E. coli strain RDEC-1 to a nonpiliated and non-adherent strain of S. flexneri. The Shigella exconjugates that inherited the ability to adhere to rabbit intestinal brush borders were also found to be piliated.

#### Pathogenesis of and Immunity to

##### Moraxella bovis in IBK

It is thought that in many cases adherence and adherence factors enhance the virulence of potential pathogens. With respect to fimbrial adhesins, there are many reports that correlate the presence of fimbriae or pili with virulence (65).

The pili of M. bovis also appear to be a principal mediator of adherence during infection of cattle and a major virulence factor in the pathogenesis of IBK.

Most investigations involving the pili of M. bovis have centered on aspects other than the functional mechanisms of adherence. The



occurrence, morphology, and structure of M. bovis pili have been studied to some extent. A rough, agar-corroding colonial morphology of M. bovis was associated with the presence of pili when organisms were examined by electron microscopy (5,66,88,91). It has been reported that M. bovis pili have a peritrichous distribution (91) with an average diameter of 6.5 to 8.5 nm (5,91) and no central core (91). Hemagglutinating activity with sheep erythrocytes also has been observed with M. bovis organisms from rough-type colonies but not from smooth colonies. This suggests that hemagglutinating characteristics are associated with the occurrence of pili (88). Gil-Turnes (28) has reported the association of hemagglutinating activity with pathogenicity of two M. bovis strains for calves.

Piliation also has been shown to contribute to the virulence of M. bovis in IBK. Only piliated M. bovis organisms were able to colonize ocular epithelial surfaces in experimental conjunctival infection in calves (8,66). The finding of an attachment advantage conferred by pili has been supported by in vivo experimental infections in mice (R. F. Rosenbusch, Abstr. Conference of Research Workers in Animal Disease, 1983, No. 17, p. 3). It also has been suggested that the hemolysin activity of M. bovis may be related to virulence. Pugh and Hughes (68) found that hemolytic M. bovis strains were pathogenic in cattle while nonhemolytic strains are not. A similar relationship was observed in mice (58). Recently, Schurig et al. (89) reported that M. bovis isolates that readily produced IBK in experimentally infected calves were able to kill phagocytes in vitro and had three plasmids. M. bovis isolates with reduced ability to produce IBK were inefficient in killing phagocytes

and had five plasmids. They suggest that the ability to destroy phagocytes may be related to virulence and be coded by plasmids present in M. bovis.

The pathogenesis of IBK has been complicated by reports that certain predisposing factors enhance the infectivity and virulence of M. bovis. Preliminary ultraviolet radiation of a particular wavelength to the eye enhanced infection and disease with M. bovis in cattle (34,45,66) and in mice (22). Application of a ragweed extract alone and in combination with UV irradiation to the eyes of mice prolonged M. bovis induced keratitis (22). In a study concerning the influence of winter environment on IBK, Kopecky et al. (46) reported that the stress of cold weather caused a more severe case with a shorter duration of clinical IBK in cattle kept outside in comparison to those housed inside. The role of another organism, Mycoplasma bovoculi, as a predisposing factor in IBK has been suggested. The presence of M. bovoculi in the eyes of cattle in naturally occurring outbreaks of IBK has been reported (20,43,48,60,86). Additional evidence demonstrated that calves infected with M. bovoculi then secondarily infected with M. bovis developed IBK while calves infected with M. bovis alone did not (20,85). The concept of predisposing factors in the pathogenesis of IBK is supported by transmission experiments using conventionally reared calves versus gnotobiotic calves (12). Three strains of M. bovis that readily established infections and produced clinical IBK in the conventional calves, colonized the eyes but failed to produce clinical IBK in all but one of the gnotobiotic calves. The authors suggested that the lack of virulence in the gnotobiotic calves might be due to their

relatively protected surrounding environment.

Histologic studies by transmission electron microscopy have provided a description of the lesion and the location of M. bovis organisms in experimentally infected conventional and gnotobiotic calves and calves with naturally occurring IBK (10). In mild cases, M. bovis was seen predominantly in subepithelial locations but in some cases was found within the epithelium at the base of the ulcer. In severe disease, bacteria were observed in the stroma of the cornea and appeared to be oriented in the direction of the collagen fibrils. It also was noted that structures resembling small microbes were seen in the stroma of some of the tissue examined. Similar histological studies in mice showed that the lesions produced by M. bovis closely resembled those in cattle (11).

By scanning electron microscopy of bovine corneas exposed in vitro to M. bovis, bacteria were observed to have a predilection for dark corneal epithelial cells, to be lying in depressions on the cell surface, and to be lodged between adjacent cells (9). An experiment with mouse corneas also showed bacteria in association with intercellular epithelial cell junctions (H. F. Trout, F. W. Piersen, and G. Schurig, Abstr. Conference of Research Workers in Animal Disease, 1981, No. 55, p. 10).

In studying the pathogenesis of M. bovis in IBK, including adherence as an essential step in the process, the ultimate goal is to protect cattle from infection. Attempts to immunize animals with various preparations of M. bovis or fractions of M. bovis have been reported. The

earliest work has been reviewed by Baptista (1). Additional work has indicated that neither a M. bovis ribosomal vaccine (76) nor a bacterin prepared from several nonhemolytic strains (74) was able to protect cattle against IBK, although with the latter, the severity of the lesion and the duration of infection was reported to be less in vaccinated calves. Vaccination of calves at birth with a M. bovis bacterin (33) or older calves with a pilus vaccine (72) demonstrated that the age at the time of vaccination did not seem to be an important determinant in altering the occurrence of IBK. Prepartum vaccination of cows also does not prevent the subsequent development of IBK in their calves (71). Calves fed colostrum from vaccinated cows were more resistant to experimental infection with M. bovis when compared to calves fed colostrum from unvaccinated cows (75).

Vaccinations of calves during an acute attack of IBK did not affect the duration of the infection (73). Webber et al. (102) reported that a M. bovis bacterin emulsified in oil and given subcutaneously to calves resulted in the lowest incidence and the shortest duration of acute IBK. This is in agreement with a previous report by Pugh et al. (70).

Studies of experimentally infected calves have correlated the appearance of specific M. bovis antibody in lacrimal secretions with the improvement of clinical IBK and the decrease in isolation of organisms from conjunctival samples (103). Kopecky et al. (47) have reported that resistance of calves to subsequent infection by M. bovis is probably mediated by a general immune response rather than a local response.

PART I. PILIATION, SURFACE HYDROPHOBICITY AND ADHERENCE PROPERTIES  
OF MORAXELLA BOVIS

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and Immunity.

PILIATION, SURFACE HYDROPHOBICITY AND  
ADHERENCE PROPERTIES OF MORAXELLA BOVIS

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## SUMMARY

Moraxella bovis strain 118F, isolated from a naturally occurring outbreak of IBK, and a UV-induced mutant of this strain, strain 118F/4-2, were studied with respect to their (1) morphology by electron microscopy; (2) ability to agglutinate human O erythrocytes; (3) adherence to suspensions of bovine corneal and conjunctival epithelial cells; and (4) surface hydrophobicity as measured by hydrophobic interaction chromatography. Piliated strain 118F exhibited a hemagglutination pattern that could not be inhibited by a variety of sugars, including D-mannose. Mutant strain 118F/4-2 was essentially nonpiliated and had little capacity for hemagglutination. A correlation was found between the adherence of radiolabeled bacteria to ocular epithelial cells and the expression of hydrophobicity. Strain 118F organisms were more adherent and more hydrophobic than strain 118F/4-2. These results suggest that hydrophobic interactions may contribute to pilus-mediated adherence of M. bovis to epithelial cells.

## INTRODUCTION

Adherence of some bacteria to epithelial cells is widely accepted as one of the first steps in the complex process of colonization of host tissues. Attachment factors called fimbriae or pili have been demonstrated as mediators of adherence in many bacterial species (17). Morphologic and hemagglutinating properties of pili have been used for descriptive characterization of these structures.

The mechanistic properties of adhesion of organisms to mammalian cells however have been more difficult to characterize and most likely include both nonspecific and specific interactions. In cell-cell interactions as a surface phenomenon, the repulsive force between the bacterial cell and the host cell, both of which are negatively charged, must be overcome. One of the mechanisms suggested to counteract this like-charged force is through hydrophobic interactions (11,15).

Moraxella bovis is the major etiologic agent associated with infectious bovine keratoconjunctivitis, an economically important ocular disease of cattle (1). In experimental infections of calves, it has been shown that only piliated strains of M. bovis are able to colonize conjunctival epithelium (3,18).

In this report, we describe the pilus-mediated properties of a M. bovis strain by i) hemagglutination, ii) hydrophobic interaction, and iii) adherence to bovine ocular epithelial cells in vitro. These properties are compared to those of an essentially nonpiliated variant of the strain.



## MATERIALS AND METHODS

## Bacteria and Growth Conditions

Moraxella bovis strain 118F was isolated, characterized, and stored as previously described (22). M. bovis 118F/4-2 was obtained by UV mutagenesis ( $100 \text{ erg/mm}^2$ ) of strain 118F followed by selection for noncorroding colonies (2). M. bovis strain Med 72 (4R) was obtained from Dr. G. W. Pugh, Jr., National Animal Disease Laboratory, Ames, IA.

Bacteria were grown on 5% bovine blood agar at  $37^\circ\text{C}$  in an atmosphere containing 10%  $\text{CO}_2$  in air for the time specified under individual test descriptions.

## Electron Microscopy

Piliation was assessed by electron microscopy of negatively stained preparations. M. bovis strain 118F (10th passage) and strain 118F/4-2 (12th passage) were grown for 21 h and gently suspended in a small amount of sterile distilled water. The cells were pelleted by centrifugation at  $150 \times g$  for 3 min and resuspended in distilled water. This step was repeated twice again and after the final centrifugation the cells were resuspended in a small amount of stain (4% phosphotungstic acid, 0.5% bovine serum albumin, and distilled water in a ratio of 1:1:8). The suspension was sprayed onto collodion-carbon coated grids and examined with a Siemens Elmiskop 101 electron microscope. Only

organisms with at least half their circumference available for evaluation were examined and the percentage of cells with pili was determined.

#### Hemagglutination

Freshly drawn blood from a variety of species was collected and preserved at 4°C in an equal volume of sterile Alsever's solution. Guinea pig blood was collected in a tube containing EDTA. The cells were washed three times in phosphate buffered saline (PBS) containing 10 mM sodium phosphate buffer, 146 mM NaCl, pH 7.5; 1% red blood cell suspensions were prepared. In some tests, 1% (wt/vol) sugars were added to the PBS.

M. bovis strain 118F (9th passage, unless otherwise specified), strain 118F/4-2 (12th passage), and strain Med 72 (4R) (13th passage) were grown for 22-24 h and suspended in ice cold PBS. Suspensions contained  $4-6 \times 10^9$  bacteria per ml. Hemagglutinating activity was determined using a microhemagglutination test system. Doubling dilutions consisting of 0.05 ml of the bacterial suspension were prepared in PBS with or without 1% sugars as appropriate. A 0.025 ml volume of 1% blood was added and the plates were shaken and allowed to incubate for 2 h at 4°C unless otherwise specified. The end point was taken as the last well showing hemagglutination and the titer was expressed as the reciprocal of this dilution.

### Radiolabeling of Bacteria

Bacteria were harvested by swab and suspended in ice cold 0.05% bovine serum albumin. An aliquot of the suspension was diluted in 10%  $\text{MgCl}_2$  to obtain a bacterial count (20). An appropriate dilution of the bacteria was made if necessary. The suspension of bacteria was centrifuged at 1000 X g for 2 min and resuspended in PBS for hydrophobic interaction chromatography (HIC) and PBS with 1mM  $\text{MgCl}_2$  for adherence assays. The cells were labeled by the addition of 20  $\mu\text{Ci/ml}$  of L-[4,5- $^3\text{H}$ ]-proline (Research Products International Corp., Mount Prospect, IL) and incubated for 60-90 sec at room temperature. This was followed by the addition of 1000X unlabeled L-proline (Sigma Chemical Company, St. Louis, MO) for 60-90 sec. The bacteria were pelleted by centrifugation at 1000 X g for 2 min and resuspended in PBS for HIC or PBS with 1mM  $\text{MgCl}_2$  for adherence assays. A second centrifugation was performed and the bacteria were resuspended in 4M NaCl buffered with 10mM sodium phosphate buffer, pH 6.8 with 1000X unlabeled L-proline for HIC or PBS with 1mM  $\text{MgCl}_2$  and 1000 X unlabeled L-proline for adherence assays.

### Hydrophobic Interaction Chromatography (HIC)

The HIC procedure was based on one described by Smyth et al. (25) with modifications. Sepharose CL-4B, phenyl Sepharose CL-4B, and octyl Sepharose CL-4B (Sigma) were washed with 4M NaCl buffered with 10mM sodium phosphate buffer, pH 6.8. The column was a Pasteur pipette, stoppered with nylon wool, and supplied with clamped tubing for draining. The gel bed was packed to a height of 30-34 mm and washed with at least

10 ml of buffered 4M NaCl before use. All reagents were allowed to equilibrate to room temperature before use. M. bovis strain 118F (9th passage), and 118F/4-2 (12th passage) were grown for 24 or 48 h and suspended in buffered 4M NaCl. Bacterial suspensions (100  $\mu$ l, containing  $2-6 \times 10^8$  bacteria) were applied to the columns, allowed to drain into the bed, and incubated for 15 min. The gel beds were washed first with 10 ml of buffered 4M NaCl, and then with 10 ml of 10mM sodium phosphate buffer. Samples of the eluates were counted to determine total number of bacteria desorbed. Percent desorption was determined as:  $(\text{Number of bacteria desorbed} \div \text{Number of bacteria applied}) \times 100$ . HIC by a radioisotope technique was performed as described with the following modifications. Radiolabeled M. bovis was applied to the columns. A 1 ml sample of each of the desorption wash eluates was centrifuged at 13,000 X g in a Fisher Micro-Centrifuge (Model 235A). The supernatant was discarded and the pellet was resuspended in 1 ml of 2% sodium dodecyl sulfate, mixed, and added to a scintillation vial containing 15 ml Aquasol-2 (New England Nuclear, Boston, MA). Radioactivity was counted with a Beckman DPM-100 liquid scintillation system. Percent desorption was determined as:  $(\text{CPM of desorbed bacteria} \div \text{CPM of bacteria applied}) \times 100$ .

#### Cell Culture

Adult bovine eyes from a local packing plant were obtained through the services of the National Animal Disease Laboratory, Ames, IA. They were transported in Hanks balanced salt solution (HBSS) with 100  $\mu$ g/ml

gentamycin and 10 µg/ml fungizone. The eyes were washed by dipping in two changes of HBSS with 0.2% Clorox (The Clorox Co., Oakland, CA) followed by a wash in HBSS. Corneas were dissected intact and split through the stroma by gently pulling apart the tissue (7). The inner stroma and endothelial cell layer were discarded. The outer stroma and epithelial layer were cut into small pieces and explanted under liquid in flasks containing Eagles minimal essential medium (MEM) supplemented with 10% fetal calf serum, 20 ng/ml epidermal growth factor (Collaborative Research, Inc., Waltham, MA), 0.1nM cholera toxin (Sigma), 0.4 µg/ml hydrocortisone (Sigma), 0.02nM triiodothyronine (Sigma), ITS Premix (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium; Collaborative Research), 50 µg/ml gentamycin, 0.06 mg/ml penicillin, 0.1 mg/ml kanamycin, and 0.1 mg/ml streptomycin. Small pieces of conjunctival tissue were snipped from the eyes and explanted in flasks containing the medium described above plus 10 µg/ml fungizone. The entire cornea or approximately ten pieces of conjunctiva from an individual eye were allotted to a flask with 20 ml of medium. Cell cultures were kept at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 3-7 days. The epithelial cells were harvested by centrifugation at 150 X g for 5 min. The cell pellet was resuspended in MEM and adjusted to 1 X 10<sup>6</sup> cells per ml. Immediately prior to use in an adherence assay, the cells were centrifuged at 150 X g for 5 min and resuspended in PBS with 1mM MgCl<sub>2</sub>.

### Adherence Assay

The adherence assay was a modification of the one described by Izhar et al. (9). Suspensions of bacteria from 22-24 h cultures were radiolabeled. Epithelial cell suspensions (200  $\mu$ l;  $2 \times 10^5$  cells) were incubated with radiolabeled bacteria (100  $\mu$ l;  $2 \times 10^8$  bacteria) for 45 min at 37°C in a moist environment with agitation at intervals. The reaction was terminated by the addition of 2 ml saline and centrifuged at 1000 X g for 3 min. The supernatant was discarded. The pellet, containing some nonadhered bacteria and cells, was subjected to a discontinuous density gradient using Percoll (Sigma). The Percoll stock solution and further dilutions were made with NaCl according to the information supplied by Pharmacia (19). The pellet was mixed with 0.5 ml of 40% Percoll, followed by layering with 2 ml of 30% Percoll, and 0.5 ml of 10% Percoll. The gradient was centrifuged at 500 X g for 15 min at room temperature. With these conditions the cells banded with the 10% layer and the nonadhered bacteria layered at the 30-40% interface. A Pasteur pipette was used to collect the top 0.6 ml of the gradient; this was diluted to a total volume of 1 ml with saline. A 500  $\mu$ l sample was transferred to a scintillation vial with 5 ml Ready Solv HP/b (Beckman Instruments, Inc., Fullerton, CA). Radioactivity was counted using a Beckman DPM-100 liquid scintillation system. A control tube containing only radiolabeled bacteria and buffer was similarly treated as described above. The radioactivity counted in the control tube probably represents free labeled proline and the CPM determined was

subtracted from that detected in tubes containing both cells and bacteria.

#### Statistical Analysis

An analysis of variance procedure was performed to determine significant differences between adherence of M. bovis strains.

## RESULTS

M. bovis Piliation

By electron microscopy, M. bovis strain 118F was greater than 96% piliated. A typical organism is shown in Fig. 1. The diameter of pili ranged from 4.4 to 7.7 nm, with an average diameter of 5.5 nm. The number observed per organism in these preparations ranged from one to ten pili. Although the pili had a peritrichous distribution, the majority were most evident at the poles of the bacterium. For M. bovis strain 118F/4-2, less than 7% of the organisms demonstrated pili when examined by electron microscopy. The pili that were observed resembled those of strain 118F.

## Hemagglutination

M. bovis strain 118F hemagglutinated all species of erythrocytes tested (Table 1). Exceptionally strong activity was seen with guinea pig, porcine, and human type O red blood cells. Human type O, the most conveniently attained blood for our laboratory, was used for subsequent assays. Because of the potent hemolytic properties associated with strain 118F and 118F/4-2, testing was performed at 4°C which retarded hemolysin activity. At room temperature, these strains caused lysis of the erythrocytes before any hemagglutination could be detected.

The addition of a variety of sugars to the buffer used in the assay did not significantly alter the hemagglutination titer of strain



Figure 1 Negatively stained preparation of M. bovis 118F  
showing typical morphology of pili.  
Bar, 500 nm.

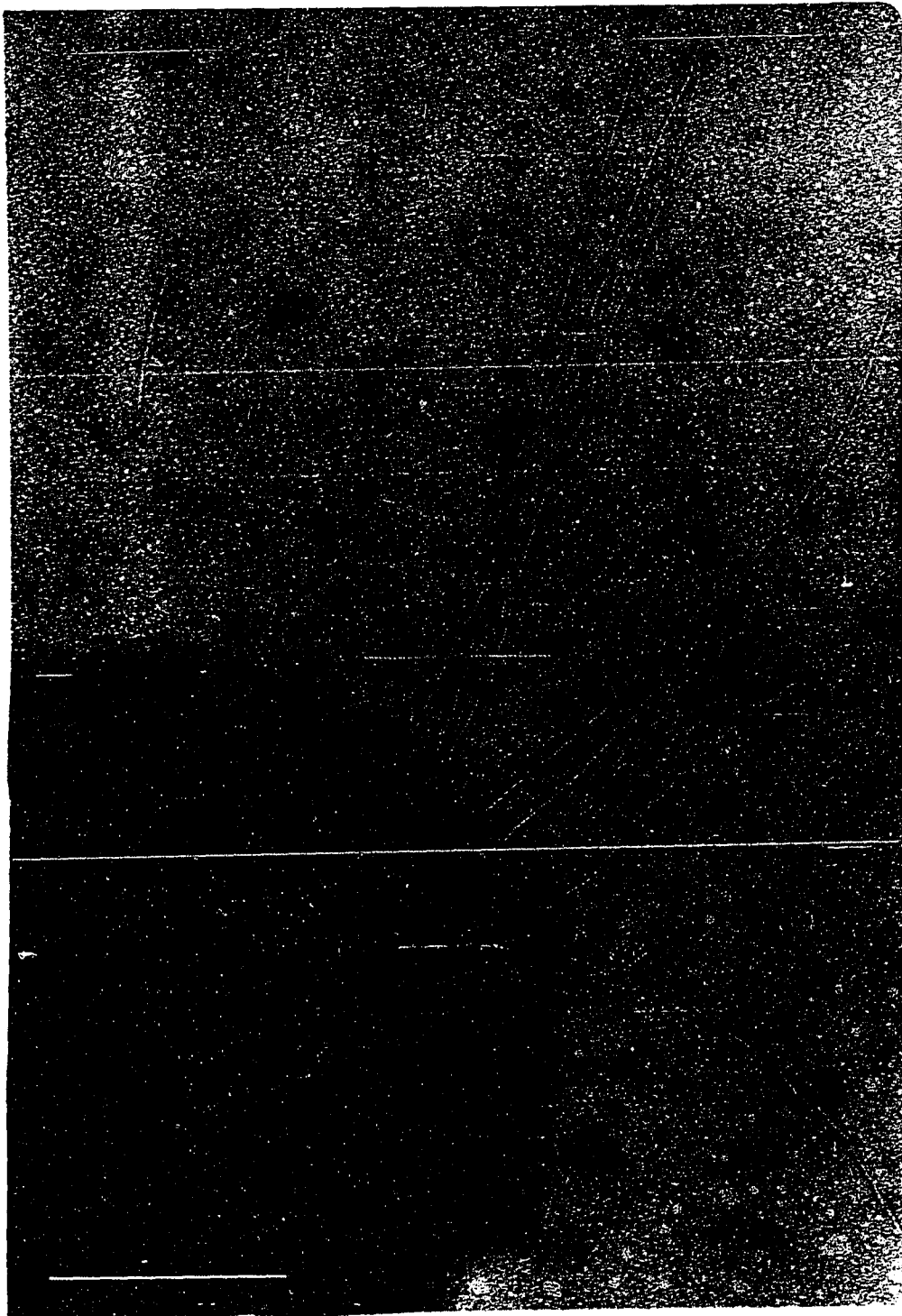


Table 1. Hemagglutinating activity of M. bovis strain 118F<sup>a</sup> to erythrocytes (RBC) from various species

Species of RBC	HA activity <sup>b</sup>
Ovine	+
Bovine	+
Equine	+
Guinea pig	++
Chicken	+
Chick (1 day old)	+
Porcine	++
Human, type 0	++

<sup>a</sup> Modifications to procedure described: M. bovis (7th passage) was grown for 48 h and suspension represented  $1 \times 10^9$  bacteria per ml; the bacteria were not serially diluted.

<sup>b</sup> Activity rating: +, positive, moderate activity; ++, positive, strong activity. Represents the estimate of three determinations.

118F (Table 2). Strain 118F/4-2, an essentially nonpiliated, hemolytic variant of 118F, did not demonstrate hemagglutinating activity beyond the first dilution whether or not sugars were added to the buffer (Table 2). The hemagglutination of strain MED 72 (4R), a piliated, nonhemolytic strain, was similar to 118F; activity was demonstrated both in the presence and absence of sugars (Table 2). Since Med 72 (4R) was nonhemolytic to erythrocytes, the assay was also performed at room temperature and at 37°C. At these temperatures, the hemagglutinating activity decreased with the highest titer equal to 4.

When the bacterial suspensions of strain 118F and Med 72 (4R) were heated to 60°C for 30 min prior to use in the assay, the ability to

Table 2. Hemagglutination activity of three strains of M. bovis in the presence of 1% sugars in phosphate buffered saline

Sugar	Hemagglutination Titer by Strain (Temp <sup>o</sup> C)				
	118F(4 <sup>o</sup> ) <sup>a</sup>	118F/4-2(4 <sup>o</sup> ) <sup>a</sup>	Med72(4R)(4 <sup>o</sup> ) <sup>b</sup>	Med72(4R)(22 <sup>o</sup> ) <sup>b</sup>	Med72(4R)(37 <sup>o</sup> ) <sup>b</sup>
PBS (no sugar)	16	<2	16	2	2
D-mannose	16	<2	32	2	<2
D-galactose	32	<2	16	2	2
Sucrose	16	<2	16	2	2
Dextrose	16	2	32	2	2
D-xylose	16	2	16	2	2
Raffinose	16	2	16	4	2
Rhamnose	32	2	8	2	2
Lactose	16	2	8	4	2
αα-trehalose	16	2	8	4	2
L-arabinose	64	2	8	4	2
Maltose	8	<2	8	2	2

<sup>a</sup> Average of 3 determinations.

<sup>b</sup> Average of 2 determinations.

hemagglutinate the erythrocytes was not decreased when tested at 4°C in the presence and absence of D-mannose.

#### Hydrophobic Interaction Chromatography

Suspensions of piliated strain 118F and essentially nonpiliated strain 118F/4-2 were chromatographed in the presence of 4M NaCl on Sepharose CL-4B and on octyl and phenyl Sepharose. The results are shown in Table 3. Strain 118F adsorbed strongly to both octyl and phenyl Sepharose in the presence of 4M NaCl. This is indicated by the low percentage of desorption at this high salt concentration; only 5% of the suspension applied was desorbed from octyl Sepharose and 1.9% from phenyl Sepharose. Lowering the ionic strength did not enhance the desorption to any extent on either of the hydrophobic gels. Interaction of strain 118F with Sepharose CL-4B was negligible, as indicated by the high percent desorption with 4M NaCl. This indicates that retention of 118F was due mainly to the hydrophobic octyl or phenyl groups coupled to the Sepharose gel.

In contrast, strain 118F/4-2 was more readily desorbed in the presence of 4M NaCl: 79% desorption on octyl Sepharose and 49.6% on phenyl Sepharose. The combined percent desorption of 118F/4-2 from both the buffered 4M NaCl eluate and the phosphate buffer eluate was comparable on all the gels: 79.7% for octyl Sepharose, 70.1% for phenyl Sepharose, and 80.3% for Sepharose CL-4B. This suggests that strain 118F/4-2 demonstrates minimal, if any, hydrophobic properties.

Table 3. Percent desorption of two strains of M. bovis from Sepharose gels

Strain	Desorption wash solution	% Desorption		
		octyl Sepharose	phenyl Sepharose	Sepharose CL-4B
118F	Buffered 4M NaCl	5.05±5.1 <sup>a</sup> (14.1) <sup>b</sup>	1.9±1.9 <sup>a</sup> (19.8)	65.2±19.0 <sup>c</sup> (92.5)
	Phosphate buffer	0.4±0.2 (9.8)	6.8±0.2 (18.0)	8.6±12.0 (6.6)
118F/4-2	Buffered 4M NaCl	79.0±28.7 (32.8)	49.6±17.0(41.4)	75.7±3.4 (88.4)
	Phosphate buffer	0.7±0.7 (1.2)	20.5±11.0(12.2)	4.6±1.1 (0.4)

<sup>a</sup>Values without parentheses represent mean of 3 determinations ± SD; by counting technique.

<sup>b</sup>Values in parentheses were determined by HIC with radiolabeled organisms and represent one determination.

<sup>c</sup>Values without parentheses represent mean of 2 determinations ± SD; by counting technique.

Results from HIC with radiolabeled M. bovis (Table 3 parentheses) substantiated the data collected with HIC by the counting technique.

These results demonstrated that adsorptive capacity of strain 118F to the gels was enhanced by the addition of the nonpolar octyl and phenyl groups coupled to the Sepharose gel. The fact that strain 118F adhered more strongly to both octyl and phenyl Sepharose than did strain 118F/4-2, suggested that pili may mediate the hydrophobic interactions between nonpolar groups.

#### Bacterial Adherence

Parameters for bacterial adherence to intact corneal and conjunctival epithelium were evaluated to optimize the adherence assay. Incubation of bacterial cells to epithelial cells at a ratio of 1000:1, and reaction times of 45 min at a temperature of 37°C resulted in optimal adherence in that model adherence system (data not shown). These conditions were applied to the adherence system described in this text. Under the conditions described for cell culture, the epithelial cells grew as individual cells or small clusters of cells and remained suspended in the medium.

The epithelial cell assay system was used to study the adherence capacity of M. bovis strains 118F and 118F/4-2 to both bovine corneal and conjunctival cells. The results are shown in Table 4. There was a significant difference in adherence between strain 118F and 118F/4-2 to both corneal ( $p < 0.05$ ) and conjunctival ( $p < 0.005$ ) cells. This was

Table 4. Adherence of two M. bovis strains to bovine corneal and conjunctival epithelial cells grown in cell culture

Strain	<u>M. bovis</u> per cell <sup>a</sup>	
	Cornea	Conjunctiva
118F	0.98±0.54 <sup>b</sup>	1.46±0.22 <sup>c</sup>
118F/4-2	0.25±0.61	0.20±0.29

<sup>a</sup>Values represent mean ± SD of 8 measurements.

<sup>b</sup> $p < 0.05$ .

<sup>c</sup> $p < 0.005$ .

interpreted to mean that adherence in this assay was mainly evaluating pilus-mediated adhesion of bacteria to epithelial cells.



## DISCUSSION

Results from electron microscopic observations on pili morphology of M. bovis strain 118F were similar to that described for several other isolates of M. bovis (24) with differences seen only in the diameter of the pili. The average diameter of pili from electron microscopy using thin sectioning and chromium shadowing techniques, was reported to be 6.5-8.5 nm (24). Our preparations of M. bovis 118F, stained with phosphotungstic acid and sprayed onto grids, resulted in an average diameter of 5.5 nm.

Correlation between piliation and hemagglutinating activity in strains 118F and 118F/4-2 was also comparable to that previously reported for other M. bovis strains (23). Additional testing using temperature modifications and Med 72 (4R), hemagglutinating activity was easily detected at 4°C, but the activity was lower at 22°C or 37°C. In contrast, the heating to 60°C did not decrease the activity. These observations might indicate that at 22°C or 37°C, an enzymatic activity is present that may digest or denature the hemagglutinating adhesin, while at 60°C the enzymatic activity is destroyed and the hemagglutinating adhesin remains stable.

Investigations of surface hydrophobicity have demonstrated a positive association between hydrophobicity and the possession of adhesins in a number of bacterial species. For example, it has been suggested that both M protein and lipoteichoic acid contribute to the hydrophobic properties of Streptococcus pyogenes. Studies with two group A Streptococcal strain pairs showed that M protein positive variants exhibited

hydrophobic characteristics which M protein negative variants lacked (28). In like manner, correlation between possession of pili and the enhanced binding of organisms possessing pili to hydrophobic gels has been readily demonstrated for many strains of E. coli (10,12,16,25,28), Streptococcus sanguis FC-1 (6) and Yersinia enterocolitica (5). With E. coli some reports indicate that there is a positive correlation between the ability to bind to hydrophobic gels and mannose resistant hemagglutinating pili found in porcine (12,25,29), bovine (12,29), and human (12,25,29) isolates. Others have demonstrated that E. coli with mannose sensitive pili were more hydrophobic than those with mannose resistant pili (10,16).

Exceptions to the pattern between the association of pili and surface hydrophobicity have been noted however with other bacterial species. Neisseria gonorrhea (26) and Neisseria meningitidis (27) fimbriae did not seem to contribute a distinct advantage in the ability to interact with hydrophobic gels and a nonfimbriated variant of Serratia marcescens was more hydrophobic on octyl Sepharose than the fimbriated organism (8).

Our findings with M. bovis indicate that mannose resistant hemagglutination and hydrophobic binding properties correlate with the expression of pili.

The relationship between hydrophobic interaction as a mechanism of adhesion between two surfaces, and the adherence of bacteria to eukaryotic cells has been studied with a variety of organisms. An association between positive hydrophobic characteristics and adherence

to mammalian cells in vitro has been demonstrated for E. coli (1,29), Salmonella typhimurium (14), Acinetobacter calcoaceticus (21), and S. pyogenes (21). Culture conditions for enhancing the expression of hydrophobic characteristics in A. calcoaceticus has promoted adherence to epithelial cells (21).

The mediators of hydrophobic and adherence properties in organisms have been inferred by investigations involving the expression of certain adhesins by the bacteria and lack of expression in other bacteria. Mutants of E. coli and S. typhimurium with decreased surface lipopolysaccharide demonstrated increased hydrophobicity and adherence to cells in vitro in contrast to nonmutant strains (14). Association of pili with hydrophobic and adhesive properties has also been suggested for E. coli (29).

Results from the adherence of M. bovis to corneal and conjunctival cells suggest that adherence of M. bovis is at least in part mediated by pili. The findings that both hydrophobic characteristics and adherence properties were expressed in M. bovis strain 118F and were negligible in strain 118F/4-2 also suggest that pili contribute to these properties of M. bovis strain 118F. It is not likely that hydrophobic interactions alone initiate adherence and colonization of M. bovis to ocular tissues in vivo. However, the evidence presented in this report indicates that hydrophobic interactions may be one of the mechanisms through which M. bovis adhere to epithelial cells.

In summary, the data presented here indicate a correlation between the expression of pili, hemagglutinating properties, surface

hydrophobicity, and the adherence to ocular epithelial cells in vitro by M. bovis 118F. The inference that hemagglutination, hydrophobic interactions and adherence is, at least in part, mediated by pili is based on the evidence that M. bovis 118F exhibit these properties while M. bovis 118F/4-2 do not.

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PART II. IN VITRO ADHERENCE OF MORAXELLA BOVIS TO INTACT CORNEAL  
AND CONJUNCTIVAL EPITHELIUM

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IN VITRO ADHERENCE OF MORAXELLA BOVIS  
TO INTACT CORNEAL AND CONJUNCTIVAL EPITHELIUM

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## SUMMARY

An in vitro assay is described using radiolabeled Moraxella bovis for studying adherence to intact bovine corneal and conjunctival epithelial surfaces. The assay was optimized for time (45 min) and for the ratio of epithelial cells to bacteria (1:1000) that demonstrated a significant difference in adherence between M. bovis strain 118F, a piliated organism and a nonpiliated variant, strain 118F/4-2. Adherence of these organisms correlated with previous pathogenicity studies involving experimental infection of calves. Scanning electron microscopy of tissues treated in the assay revealed a predilection of M. bovis for dark epithelial cells and for association with depressions in the tissue surface. This assay technique is discussed in comparison with other in vitro adherence assay methods.

## INTRODUCTION

Moraxella bovis is the organism commonly regarded as the primary etiologic agent in infectious bovine keratoconjunctivitis (IBK), an important ocular disease of cattle (1). As with other mucosal surfaces, organisms colonizing ocular epithelium must keep from being swept away in order to establish an infection in the host. Adherence of bacteria to epithelial cells may be such a way to evade clearance and is accepted as one of the first steps in the process of colonization.

Many adherence assays utilize suspensions of epithelial cells or monolayers of tissue culture cells. Here, we report an in vitro assay for studying the adherence of M. bovis to intact bovine corneal and conjunctival epithelium.

## MATERIALS AND METHODS

Moraxella bovis

M. bovis strain 118F was isolated from a naturally occurring infection of IBK and was characterized and stored as previously described (7). M. bovis 118F/4-2 was obtained by UV mutagenesis ( $100 \text{ erg/mm}^2$ ) of strain 118F. Selected characteristics of these organisms are shown in Table 1.

Table 1. Selected characteristics of M. bovis strains used in this report

Parameter	<u>Moraxella bovis</u>	
	118F	118F/4-2
Source	Isolated from a herd with IBK	Variant produced by UV mutagenesis
Hemolysin activity on bovine blood agar	yes	yes
Piliation	>96%	<7%
Ability to produce IBK in calves by experimental inoculation	yes <sup>a</sup>	no <sup>b</sup>

<sup>a</sup>Reported in reference 7.

<sup>b</sup>R. F. Rosenbusch, Iowa State University, unpublished data.

### Cattle Eyes

Adult cattle eyes were obtained from a packing plant through the services of the National Animal Disease Laboratory, Ames, IA. They were transported in Hanks balanced salts solution (HBSS) with 100 ug/ml gentamycin and 10 ug/ml amphotericin B.

### Preparation of Radiolabeled Bacteria

M. bovis strain 118F (9th passage) and 118F/4-2 (12th passage) were grown on 5% bovine blood agar plates for 22-24 h at 37°C in an atmosphere containing 10% CO<sub>2</sub> in air. Bacterial growth was gently suspended in 1 ml of ice-cold 0.05% bovine serum albumin. An aliquot of the suspension was diluted in 10% MgCl<sub>2</sub> to obtain a bacterial count. The suspension of bacteria was centrifuged at 1000 X g for 2 min and resuspended in phosphate buffered saline (PBS/Mg) containing 10mM Na<sub>2</sub>HPO<sub>4</sub>, 10mM NaH<sub>2</sub>PO<sub>4</sub>, 146mM NaCl, and 1mM MgCl<sub>2</sub>, at pH 7.4. The cells were labeled by the addition of 20 µCi/ml of L-[4,5-<sup>3</sup>H] proline (Research Products International Corp., Mount Prospect, IL) and incubated for 60-90 sec at room temperature; this was followed by the addition of 1000X unlabeled proline (Sigma Chemical Co., St. Louis, MO) for 60-90 sec. The bacteria were sedimented by centrifugation at 1000 X g for 2 min and resuspended in PBS/Mg. A second centrifugation was performed and the bacteria were resuspended in PBS/Mg with 1000X unlabeled proline.

### Adherence Assay of Bacteria to Epithelial Surfaces

Corneas were dissected and split through the stromal layer by gently pulling apart the tissue while holding it with forceps (4). The inner stroma and endothelial cell layer were discarded. The outer stroma and epithelial layer were cut into small pieces and a piece was placed on a flat plastic surface with the epithelial cell layer facing upwards. A second matching plastic surface containing three holes (diameter = 3 mm, center to center distance = 4 mm) was placed on top and held with two screws. Conjunctival epithelium was prepared by cutting bulbar conjunctival pieces within 10-20 mm of the cornea and placing it between similar plastic surfaces so that the epithelial cell layer was exposed in the holes. The holes were filled with Eagles minimal essential medium (MEM) until use in the adherence assay, at which time the MEM was removed and the tissue was washed once with PBS/Mg.

The adherence assay was a modification of one described by Izhar et al. (5). Radiolabeled bacteria ( $20 \mu\text{l}$ ;  $2 \times 10^8$  bacteria/ml for corneas and  $5 \times 10^8$  bacteria/ml for conjunctivas) were added to the holes and the apparatus was incubated at  $37^\circ\text{C}$  in a moist environment for 45 min, unless otherwise specified. The reaction was terminated by the removal of the unattached bacteria. For corneas, the tissue was removed from the apparatus and washed in three changes of saline. For conjunctivas, the top plastic surface only was removed and the tissue was washed in three changes of saline. The areas exposed to the labeled bacteria were cut out and placed in a tube with 1 ml of 2% sodium dodecyl sulfate and heated at  $100^\circ\text{C}$  for 15 min. The epithelial

layers were solubilized under these conditions and a 200  $\mu$ l sample was transferred to scintillation vials containing 5 ml of Ready Solv HP/b (Beckman Instruments, Inc., Fullerton, CA). Radioactivity was counted using a Beckman DPM-100 liquid scintillation system.

#### Scanning Electron Microscopy

The tissues were prepared and incubated with unlabeled bacteria as described for the adherence assay. After washing, the tissue was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for several hours. The tissue was rinsed and then stored in buffer until further processing. Areas exposed to the bacteria were cut out and post-fixed in 1% osmium tetroxide for 1 h. They were then rinsed in buffer, dehydrated in a series of alcohols and Freon 113, critical point dried using CO<sub>2</sub> as the transition fluid and sputter coated with a gold-palladium (60-40) target (Polaron E5100) Sputter Coater). The tissues were examined with a JSM-35 scanning electron microscope at an operating voltage of 20 kV.

## RESULTS

## Parameters for Optimal Adherence

The effect of time on adherence of M. bovis strain 118F to adult corneal epithelium is shown in Table 2. Maximal adherence occurred within 30 min of incubation and remained steady thereafter until at least 90 min. For subsequent assays, a 45 min incubation time was used. No significant difference in adherence at 25°C or 37°C was noted with 45 min incubation time (data not shown).

Table 2. Effect of time on the rate of adherence of M. bovis 118F to adult corneal epithelium. Assays were performed with  $1 \times 10^8$  organisms per adherence well

Incubation time (min)	Adherence ( $\times 10^3$ <u>M. bovis</u> per cell)
0	0.4 $\pm$ 0.2 <sup>a</sup>
10	2.1 $\pm$ 0.5
30	3.1 $\pm$ 1.9
50	2.9 $\pm$ 0.5
70	3.1 $\pm$ 1.5
90	3.0 $\pm$ 1.0

<sup>a</sup>Results represent mean  $\pm$  SD from four experiments.

Table 3 shows the results of incubating various concentrations of the two strains of M. bovis in an adherence assay with adult corneal



epithelial tissue. The optimal concentration of M. bovis to use in further assays was evaluated as that concentration which gave the most significant difference in adherence between piliated strain 118F and the essentially nonpiliated variant 118F/4-2. At all concentrations, 118F/4-2 adhered less well than strain 118F. The difference in adherence between the strains was most significant ( $p < 0.01$ ) when  $4 \times 10^6$  bacteria

Table 3. Adherence of M. bovis strains to adult corneal epithelium using three concentrations of bacteria

Concentration/adherence well	Adherence ( <u>M. bovis</u> per cell) <sup>a</sup>	
	118F	118F/4-2
$4 \times 10^5$	139±49	103±39
$4 \times 10^6$	211±71	86±34 <sup>b</sup>
$4 \times 10^7$	765±160	445±172 <sup>c</sup>

<sup>a</sup>Results represent mean ± SD from three corneas. Corneas were blocked and each treatment was represented in duplicate.

<sup>b</sup>Significant difference in adherence when compared to the corresponding value for strain 118F;  $p < 0.01$ .

<sup>c</sup>Significant difference in adherence when compared to the corresponding value for strain 118F;  $p < 0.05$ .

were added to the adherence well. This represented a ratio of epithelial cells: bacteria of 1:1000. The number of epithelial cells per adherence

well was estimated from scanning electron micrographs of tissue treated as in the adherence assay, but without the addition of bacteria, through the washing steps. It was then fixed and processed for scanning electron microscopy as has been described above. It was calculated that there were approximately  $4 \times 10^3$  corneal epithelial cells per adherence well and  $1 \times 10^4$  conjunctival epithelial cells per well.

#### Scanning Electron Microscopy

Figure 1 shows the adherence of M. bovis strain 118F to bovine corneal epithelium under optimized conditions. Bacteria adhere both to the epithelial surface as well as to other microorganisms. Adherence of strain 118F to bovine conjunctival epithelium is demonstrated in Figure 2.

Many bacteria appear to be attached at their poles to epithelial cells and to be associated with crevices in the epithelial surface (Fig. 1 and 2). Most often the characteristic localization of M. bovis was on epithelial cells which lacked microvilli or dark cells; few bacteria are seen associated with microvilli-abundant cells or light cells (Fig. 2).

Figure 1 Adherence of M. bovis 118F to corneal epithelium.

Bacteria seem to attach at their poles (arrowhead)  
and to be associated with depressions in the tissue  
surface (arrow).

Bar, 5 $\mu$ .

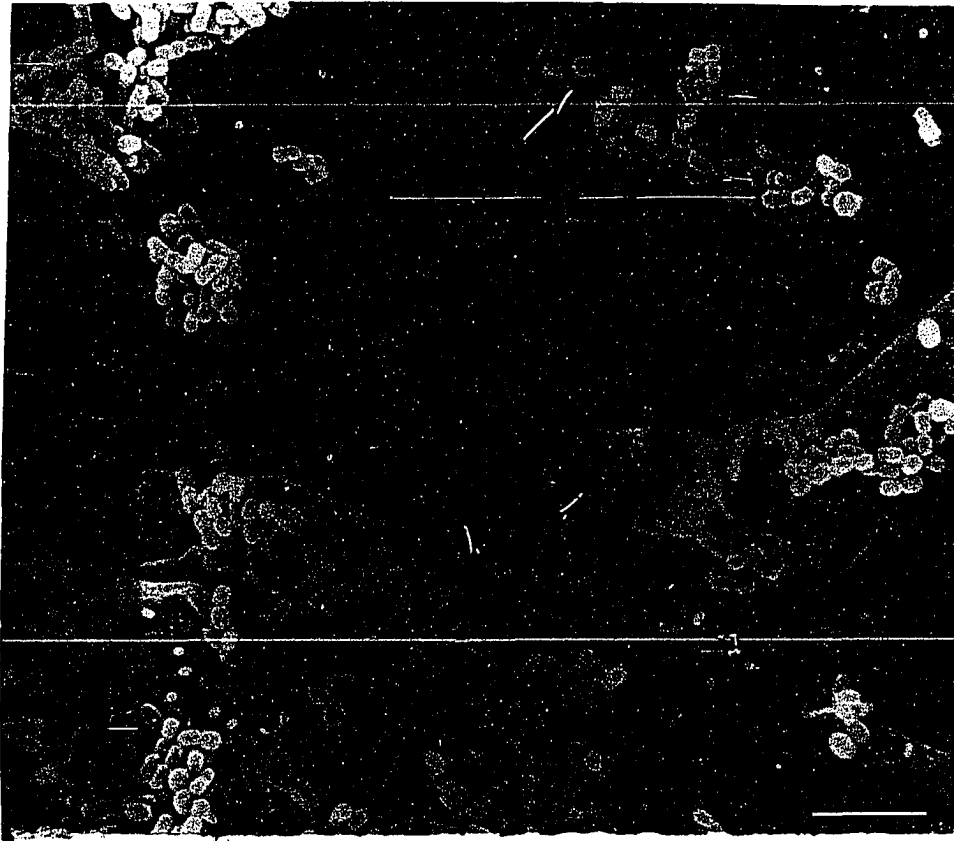
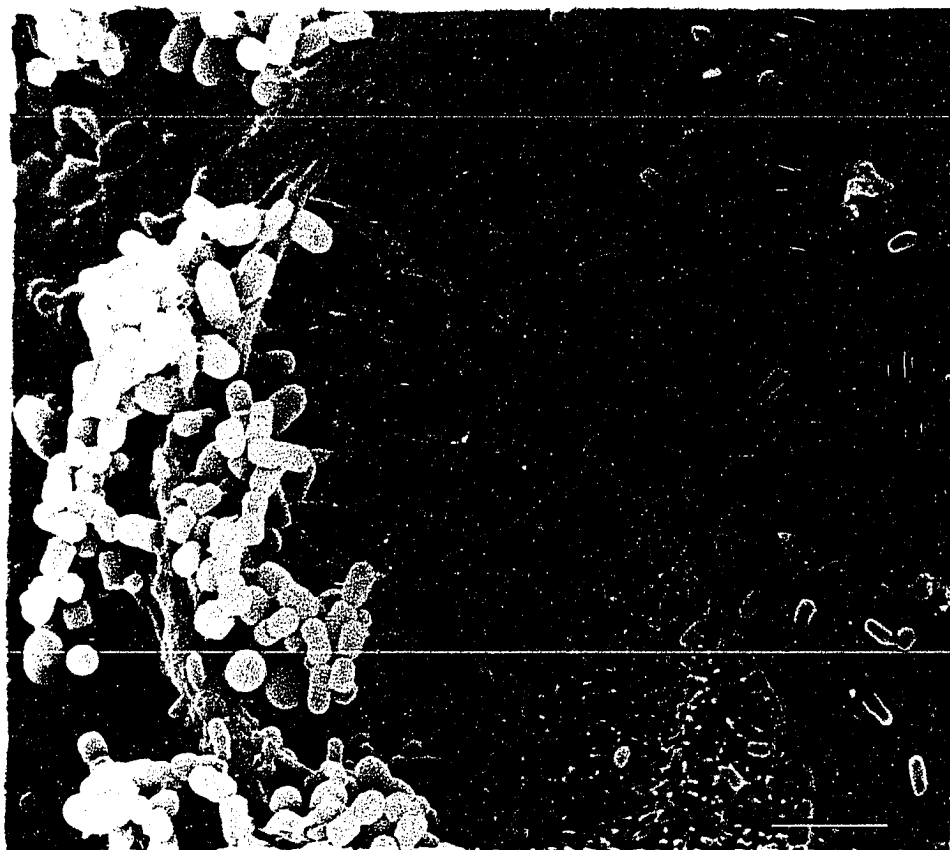


Figure 2 Adherence of M. bovis 118F to conjunctival epithelium.

Bacteria seem to have a predilection for dark cells  
and a lack of association with light epithelial cells.

Bar, 5 $\mu$ .



## DISCUSSION

The adherence assay described in this report has several advantages over other procedures. First, the difficulty and time needed to obtain ocular epithelial cells in tissue culture is eliminated. Second, it provides a means whereby several experimental treatments can be applied to the tissue obtained from one eye. This can substantially reduce the number of individual eyes needed to provide the necessary number of replications for accurate evaluation of results by statistical analysis. We have found, using an analysis of variance procedure, that the variability among corneas from individual cattle is significant and warrants caution in interpreting results. If all experimental protocols can be performed on tissue from one donor, then the variability among individuals can be accounted for in determining differences between experimental treatments.

In preliminary attempts to develop an in vitro adherence assay that would resemble as closely as possible adherence to epithelial surfaces in vivo, we incubated bacteria with small explants of corneal tissue without the aid of the plastic instrument described in this report. Upon microscopic examination of these explants, we found that M. bovis adhered to the cut edges of the explants in greater numbers than to epithelial surface cells. Because these "edges" most likely are not available to M. bovis during colonization of intact eyes, we felt that interpretation of results from experiments using this system would be erroneous.

The scanning electron micrographs demonstrating M. bovis adherence obtained by the assay described here are similar to those previously described in cattle (3) and in mice (H. F. Trout, F. W. Pierson, and G. Schurig, Abstr. Conference of Research Workers in Animal Disease, 1981, No. 55, p 10). With respect to the cornea, M. bovis seem to attach more to dark epithelial cells than to light epithelial cells. With both corneal and conjunctival tissue, many of the bacteria, singularly and in clumps, were found wedged into the epithelial surface and along intercellular junctions. Because of the predilection for certain cell types and areas of epithelium, it would seem that the use of intact epithelial surfaces best represents M. bovis adherence in cattle.

The present study was designed to develop a model to study the adherence of M. bovis to ocular epithelial cells. We have shown that the assay described in this report can indeed measure adherence of M. bovis. The differences in adherence between strain 118F and 118F/4-2 suggests that adherence of M. bovis is mediated by pili. These data correlate with observations that pili are required for infectivity and/or pathogenicity of M. bovis in calves (2,6). This assay will provide a basis for further investigations regarding M. bovis adherence properties.

We feel that the technique reported here may be applicable for studies of mammalian ocular epithelium both as an adherence assay for microorganisms as well as an assay for other pathogenic mechanisms.



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PART III. STUDY OF MYCOPLASMA BOVOCULI PREINFECTION ON THE  
IN VITRO ADHERENCE OF MORAXELLA BOVIS TO BOVINE CORNEAL  
AND CONJUNCTIVAL EPITHELIAL CELLS

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STUDY OF MYCOPLASMA BOVOCULI PREINFECTION ON THE  
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## SUMMARY

In vitro assays were used to evaluate the adherence of Moraxella bovis to intact bovine corneal and conjunctival epithelium and to suspensions of bovine corneal and conjunctival epithelial cells. Adherence of M. bovis to tissue and suspensions of cells that had been previously infected with Mycoplasma bovoculi was compared with adherence to uninfected tissue and cells. M. bovoculi preinfection decreased the number of adherent M. bovis organisms but the difference in comparison to M. bovoculi uninfected tissue and cells could not be considered to be significant. The results are discussed in relation to microbial interactions in disease processes.

## INTRODUCTION

Moraxella bovis is the organism most commonly associated with infectious bovine keratoconjunctivitis (IBK), an economically important ocular disease of cattle (1). The ability of predisposing factors to enhance the colonization of M. bovis and disease in cattle has been suggested (1). Recent reports show that ultraviolet radiation of calves (12), exposure to ultraviolet radiation and ragweed extracts in mice (6), and Mycoplasma bovoculi infection of calves (19) do indeed enhance the colonization and pathogenesis of M. bovis in experimentally infected animals.

Adherence of certain bacteria to epithelial cells is regarded as one of the first steps in the complex process of colonization. It has already been demonstrated for many organisms that fimbriae or pili mediate adherence (16). Likewise, it has been reported that only piliated strains of M. bovis are able to colonize conjunctival epithelium of experimentally infected calves (2,17).

In this report, data are obtained regarding the interrelationship of M. bovoculi infection and adherence of M. bovis to corneal and conjunctival epithelium.

## MATERIALS AND METHODS

### Microorganisms

Isolation and characterization of Mycoplasma bovoculi has been previously described (20). Broth culture aliquots of twice cloned organisms were stored at  $-70^{\circ}\text{C}$  and used as inoculum at the 8th passage level. Strain 118F of Moraxella bovis was isolated, characterized, and stored as previously described (19).

### Calves

Calves were obtained from local dairy herds. One calf was taken from a closed herd of Hereford cattle. They were held individually in isolation rooms and fed antibiotic-free calf ration. All were culturally negative for M. bovoculi and M. bovis prior to experimentation. Calves were killed by intravenous injection with an overdose of sodium pentobarbital (Fort Dodge Laboratories, Inc., Fort Dodge, IA).

### Cell Culture

Adult bovine eyes from packing plant cattle were obtained through the services of the National Animal Disease Laboratory, Ames, IA. They were transported in Hanks balanced salt solution (HBSS) with 100  $\mu\text{g/ml}$  gentamycin and 10  $\mu\text{g/ml}$  fungizone. Corneas were dissected intact and split through the stroma by gently pulling apart the tissue (7). The inner stroma and endothelial cell layer were discarded. The outer stroma

and epithelial layer were cut into small pieces and explanted in flasks containing Eagles minimal essential medium (MEM) and supplemented with 10% fetal calf serum, 20 ng/ml epidermal growth factor (Collaborative Research, Inc., Waltham, MA) and 0.1nM cholera toxin (Sigma Chemical Company, St. Louis, MO), 0.4 µg/ml hydrocortisone (Sigma), 0.02nM triiodothyronine (Sigma), ITS Premix (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium) (Collaborative Research), 50 µg/ml gentamycin, 0.06 mg/ml penicillin, 0.1 mg/ml kanamycin, and 0.1 mg/ml streptomycin. Small pieces of conjunctival tissue were snipped from the eyes and placed in flasks containing the medium described above plus 10 µg/ml fungizone. The entire cornea or approximately ten pieces of conjunctiva from an individual eye were allotted to a flask with 20 ml of medium. Cell cultures were kept at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air.

#### Infection of Calves with M. bovoculi

Seven days prior to use in an experiment, both eyes of designated calves were infected with M. bovoculi. A suspension of M. bovoculi inoculum was diluted with an equal volume of Friis broth; 1.5 ml of the dilute suspension containing 10<sup>4</sup> color changing units/ml was instilled into the lower conjunctival sac of each eye. The suspension was delivered with a plastic syringe without a needle. The day before the experiment, conjunctival scrapings of both eyes of each calf were

obtained and evaluated for the presence or absence of M. bovoculi by indirect fluorescence microscopy. These procedures have been described elsewhere (19).

#### Infection Of Cell Culture With M. bovoculi

A flask of cells was divided into two aliquots and placed in the medium described above without antibiotics and the fetal calf serum was increased to a 20% concentration. One of the aliquots was then infected with a 48 h broth culture of M. bovoculi (9th passage) at a volume equal to 1/50 of the volume of the flask. The other aliquot was left uninfected. The cells were allowed to grow in culture for an additional 5 days. One day prior to use in an adherence assay, a sample of the cell culture was evaluated for M. bovoculi infection by indirect fluorescence microscopy (19).

#### Preparation of Radiolabeled Bacteria

M. bovis strain 118F (9th passage) was grown on 5% bovine blood agar plates for 22-24 h at 37°C in an atmosphere containing 10% CO<sub>2</sub> in air. Bacterial growth was gently suspended in 1 ml of ice-cold 0.05% bovine serum albumin. An aliquot of the suspension was diluted in 10% MgCl<sub>2</sub> to obtain a bacterial count. An appropriate dilution of the bacteria was made if necessary. The suspension of bacteria was centrifuged at 1000 X g for 2 min and resuspended in phosphate buffered saline (PBS/Mg) containing 10mM Na<sub>2</sub>HPO<sub>4</sub>, 10mM NaH<sub>2</sub>PO<sub>4</sub>, 146nM NaCl, and



1mM  $\text{MgCl}_2$ , at pH 7.4. The cells were labeled by the addition of 20  $\mu\text{Ci/ml}$  of L-[4,5<sup>3</sup>H]proline (Research Products International Corp., Mount Prospect, IL) and incubated at room temperature for 60-90 sec. This was followed by the addition of 1000X unlabeled proline (Sigma) for 60-90 sec. The bacteria were sedimented by centrifugation at 1000 X g for 2 min and resuspended in PBS/Mg. A second centrifugation was performed and the bacteria were resuspended in PBS/Mg with 1000X unlabeled proline.

#### Adherence Assay of M. bovis to Epithelial Surfaces

The adherence assay was a modification of one described by Izhar et al. (10). Corneas were dissected and split as described above. The outer stroma and epithelial layer were cut into small pieces and a piece was placed on a flat plastic surface with the epithelial cell layer facing upwards. A second matching plastic surface containing three holes (diameter = 3 mm, center to center distance = 4 mm) was pressed on top and held with two screws. Conjunctival epithelium was prepared by cutting bulbar conjunctival pieces within 10-20 mm of the cornea and placing them between similar plastic surfaces so that the epithelial cell layer was exposed in the holes. The wells were filled with MEM until use in the adherence assay, at which time the MEM was removed and the tissue was washed once with PBS/Mg. Radiolabeled bacteria ( $20 \mu\text{l}$ ;  $2 \times 10^8$  bacteria/ml for corneas and  $5 \times 10^8$  bacteria/ml for conjunctivas) were added to the wells and the apparatus was incubated

at 37°C in a moist environment for 45 min. The reaction was terminated by removal of the unattached bacteria. For corneas, the tissue was removed from the apparatus and washed in three changes of saline. For conjunctivas, the top plastic surface only was removed and the tissue was washed in three changes of saline. The areas exposed to the labeled bacteria were cut out and placed in a tube with 1 ml of 2% sodium dodecyl sulfate and heated at 100°C for 15 min. The epithelial layers were solubilized under these conditions and a 200 µl sample was transferred to scintillation vials containing 5 ml of Ready Solv HP/b (Beckman Instruments, Inc., Fullerton, CA). Radioactivity was counted using a Beckman DPM-100 liquid scintillation system.

#### Adherence Assay of M. bovis to Cell Suspensions

Epithelial cells were harvested by centrifugation at 150 X g for 5 min. The cell pellet was resuspended in MEM and adjusted to  $1 \times 10^6$  cells per ml. Immediately prior to use in the adherence assay, the cells were centrifuged again and resuspended in PBS/Mg. The adherence assay was a modification of the one described by Izhar et al. (10). Epithelial cell suspensions (200 µl;  $2 \times 10^5$  cells) were incubated with radiolabeled bacteria (100 µl;  $3 \times 10^8$  bacteria) for 45 min at 37°C in a moist environment with agitation at intervals. The reaction was terminated by the addition of 2 ml of saline and centrifuged at 1000 X g for 3 min. The supernatant fluid was discarded. The pellet, containing some nonadhered bacteria and cells, was subjected to a

discontinuous density gradient using Percoll (Sigma). The Percoll stock solution and further dilutions were made with a saline solution according to information supplied by Pharmacia (18). The pellet was mixed with 0.5 ml of 40% Percoll, followed by layering with 2 ml of 30% Percoll, and 0.5 ml of 10% Percoll. The gradient was centrifuged at 500 X g for 15 min at room temperature. With these conditions the cells banded with the 10% layer and the nonadhered bacteria layered at the 30-40% interface. A Pasteur pipette was used to collect the top 0.6 ml of the gradient; this was diluted to a total volume of 1 ml with saline. A 500  $\mu$ l sample was transferred to a scintillation vial and radioactivity was counted as described above.

## RESULTS

Results of the adherence assays are shown in Table 1. With respect to the cornea, both assays using epithelial surfaces and suspensions of corneal cells indicate a reduction in the number of M. bovis organisms adhering per cell in the presence of M. bovoculi infection. With conjunctival cells in suspensions, M. bovoculi infection also results in a decrease in the number of M. bovis organisms per cell while with conjunctival epithelial surfaces, there is virtually no difference in adherence of M. bovis in M. bovoculi infected versus uninfected calves. However, in no case where there is a modification in adherence, is the difference considered to be significant. Because these are preliminary data with a small number of determinations, statistical significance between the relationship of M. bovoculi infection and M. bovis adherence was not evaluated. However, the data suggest that M. bovoculi does not modulate the initial adherence of M. bovis to bovine ocular epithelial cells.

Table 1. Adherence of M. bovis strain 118F to Mycoplasma bovoculi infected and uninfected cells

Tissue	<u>M. bovoculi</u>	Adherence ( <u>M. bovis</u> per cell)	
		Epithelial surface	Cell suspensions
Cornea	Uninfected	77±42 <sup>a</sup>	8.7±4.2
	Infected	44±14	3.5±1.8
Conjunctiva	Uninfected	79±56	6.4±3.6
	Infected	81±36	3.3±1.8

<sup>a</sup>Mean ± SD from 2 determinations.

## DISCUSSION

Microbial interactions in disease have been recognized for many years. Both from observational studies and from investigations involving experimentally infected animals, two or more organisms could be correlated to the manifestation of a particular pathologic condition.

Synergism between organisms in pulmonary infections has been studied to a great extent in a variety of animal species. Interaction between respiratory viruses and bacteria has been well-recognized in cattle (26). Pneumonia associated with combined mycoplasma-bacterial infections also have been reported in cattle (8). However, respiratory epithelium is not unique in the colonization and infection by interacting microorganisms. Combined infection with rotavirus and Escherichia coli produced intestinal lesions and diarrhea to a greater extent in gnotobiotic calves with mixed infections than in those with single infections (P. L. Runnels, H. W. Moon, S. C. Whipp, P. J. Matthews, and G. N. Woode, Abstr. Conference of Research Workers in Animal Disease, 1982, No. 242, p. 44. A. Torres-Medina, Abstr. Conference of Research Workers in Animal Disease, 1982, No. 243, p. 44).

The possible interaction of M. bovoculi and M. bovis in infectious bovine keratoconjunctivitis has been suggested by the isolation of both of these organisms together in naturally occurring outbreaks of IBK (5,11,13). The presence of M. bovoculi alone only leads to conjunctivitis (15,20). In addition, earlier work in our laboratory has shown that eye infections caused by M. bovoculi are prevalent among cattle in the

midwestern United States (20). This was indeed confirmed by the difficulty in obtaining M. bovoculi free calves. Transmission experiments involving experimental inoculation of M. bovoculi followed by M. bovis onto the eyes of cattle reproduced clinical symptoms similar to naturally occurring IBK (5,20). Previous work in our laboratory has shown that inoculation of calves with bovine ocular Ureaplasma sp followed by challenge with M. bovis does not result in IBK (19). This suggests that M. bovoculi may be singular in its ability to predispose the bovine eye to infection with M. bovis.

Mechanisms of interaction between M. bovoculi and M. bovis are presently unknown. Since adherence to epithelial cells is considered an initial step in the pathogenesis of many infections, it was decided that the adherence of M. bovis to bovine ocular epithelium would be investigated.

In respiratory illness studies, in vitro bacterial adherence to cells previously infected naturally or experimentally with virus have demonstrated both increased and decreased bacterial adherence depending on the species of bacteria and the types of virus utilized (3,21,23) and the time between infections (23).

Overall, in our assay, adherence of M. bovis decreased when epithelium had been previously infected with M. bovoculi. However, this decrease was not considered to be at a significant level. Although, the interaction between M. bovoculi and M. bovis, based on an adherence phenomenon, could not be demonstrated with the conditions used in our assay system, further work needs to be done to validate these results.

These results suggest that M. bovoculi may be predisposing an animal to M. bovis by mechanisms other than alteration of adherence. Factors, such as mucus or tear fluid components may be involved in the colonization of M. bovis.

Interactions of mycoplasmas with host cells to elicit or suppress host immune responses nonspecifically have been suggested (4,22). One investigation of the in vitro bactericidal activity of human leukocytes against E. coli demonstrated impairment of bacterial killing when the leukocytes were pre-incubated with Mycoplasma hominis (24). However, subsequent reports have indicated that impaired ability to kill bacteria may not represent a valid in vivo mechanism of suppression of the immune response by mycoplasma. Such reports have shown that pneumonia induced in mice by Mycoplasma pulmonis did not decrease resistance to subsequent bacterial infection by Staphylococcus aureus (9) and that Mycoplasma bovis infection in calves did not interfere with the pulmonary clearance of Pasteurella haemolytica (14).

In a recent review, Stanbridge (25) has discussed the interaction between mycoplasma and host lymphocytes; mycoplasmal organisms have been shown to stimulate both B and T lymphocytes to undergo blastogenesis in a nonspecific manner. Speculation on the relevance of such polyclonal activation has suggested the development of immunopathologic conditions or inhibition of lymphocyte function within the host (25).

Investigations on the interaction of mycoplasma and the host immune system may be needed to clarify the question of the mechanism of pre-



disposition of M. bovoculi to M. bovis colonization of the bovine eye in IBK.

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## CONCLUSIONS

This research involved the study of adherence of Moraxella bovis 118F and a UV-derived mutant 118F/4-2.

Negative stain preparations of these M. bovis strains demonstrated that 118F readily expressed pili when grown on bovine blood agar while 118F/4-2 was essentially nonpiliated. The diameter of pili ranged from 4.4 to 7.7 nm with an average diameter of 5.5 nm. The pili had a peritrichous distribution but were most prominently found at the poles of the bacteria. Hemagglutinating ability was not inhibited by a variety of sugars, including D-mannose and correlated with the degree of piliation.

Surface hydrophobicity was evaluated using hydrophobic interaction chromatography. M. bovis 118F readily adsorbed to the hydrophobic gels and was not easily desorbed. In contrast, 118F/4-2 was desorbed to a high degree.

In vitro adherence assays both to corneal and conjunctival epithelial cells in culture and to intact corneal and conjunctival epithelium from explanted tissue were developed. The assays measured the adherence of radiolabeled M. bovis 118F and 118F/4-2. In both assay systems and with both corneal and conjunctival cells, M. bovis 118F adhered in significantly higher numbers than did 118F/4-2. Comparison of M. bovis adherence between corneal and conjunctival epithelium was not determined and may be the subject of further research.

Scanning electron microscopy of M. bovis to corneal and conjunctival

epithelium revealed a predilection of M. bovis for dark epithelial cells, that is, those cells with few microvilli. Bacteria were often seen in association with depressions in the tissue surface and along inter-cellular junctions.

Both in vitro adherence assays were used to evaluate the effect of Mycoplasma bovoculi preinfection on the adherence of M. bovis. Overall, M. bovoculi infection decreased the number of adhering M. bovis organisms. However, the difference in adherence of M. bovis to M. bovoculi infected versus uninfected epithelium was not considered to be significant.

The conclusions drawn from these studies with M. bovis 118F and 118F/4-2 are the following:

1. The expression of pili correlates with hemagglutinating ability, surface hydrophobicity, and in vitro adherence to bovine corneal and conjunctival epithelial cells.

2. The data strongly suggest that hydrophobic interactions contribute to the mechanisms by which M. bovis organisms adhere to epithelial cells.

3. In contrast to recent reports of in vivo infections of calves demonstrating increased M. bovis colonization of conjunctival epithelium in M. bovoculi inoculated animals (85), interaction between M. bovoculi and M. bovis, based on an adherence phenomenon, could not be conclusively shown with the assay conditions used in this research.

Work still needs to be done in purifying and defining the specificity of the bacterial adhesin and the receptor on the epithelial cell for

M. bovis. This would significantly contribute to understanding the role of surface structures in the colonization of M. bovis. It may also help to clarify the mechanisms of M. bovis adherence. The observation from scanning electron microscopy, that M. bovis are most often associated with dark epithelial cells, may provide a key for future research and several hypotheses may be considered: (1) Based on the DLVO theory, surfaces having projections with a low radius of curvature facilitate the approach of the two bodies. From this concept, it would be expected that the light epithelial cells would be the better cell for M. bovis adherence. However, there may be projections on dark epithelial cells that have not yet been identified. (2) Dark cells may exhibit a lower negative or even a positive surface charge. It has been noted that these types of changes in surface charge facilitate bacterial adherence (40). (3) Dark epithelial cells may have areas more favorable for hydrophobic interactions with bacterial surfaces. (4) Specific bacterial receptors may be more readily exposed on dark cells. The reason(s) for the association of M. bovis with dark epithelial cells will not easily be resolved but will contribute to the knowledge of bacterial-eukaryotic cell adherence.

In addition, and even more intriguing, is the role of M. bovoculi in the pathogenesis of IBK. Research investigating further the interaction of M. bovoculi with M. bovis alone or in combination with the host immune system may be needed to understand more fully the pathogenesis of infectious bovine keratoconjunctivitis.

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